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Original article

Oral delivery of fish oil in oil-in-water nanoemulsion: development, colloidal stability and modulatory effect on *in vivo* inflammatory induction in mice

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ABSTRACT

To improve the oral absorption of fish oil and test its anti-inflammatory effect, a fish oil nanoemulsion was developed using *cis*-4,7,10,13,16,19-docosahexaenoic fatty acid as a biomarker for oral administration. The colloidal stability tests of the fish oil nanoemulsion showed an average size of 155.44 nm ± 6.46 (4 °C); 163.04 nm ± 9.97 (25 °C) and polydispersity index 0.22 ± 0.02 (4 °C), 0.21 ± 0.02 (25 °C), indicating systems with low polydispersity and stable droplets. The fish oil nanoemulsion did not alter the cell viability of the RAW 264.7 macrophages and, at a concentration of 0.024 mg/mL, was kinetically incorporated into the cells after 18 h of contact. The nanoemulsion was maintained in the gastrointestinal region for a significantly shorter period of time ($p \leq 0.05$) compared to the intake of fish oil in free form. Inflammatory tests demonstrated that nanoemulsion and fish oil showed less ($p \leq 0.05$) neutrophil infiltration after 24h of sepsis induction and there was a significant reduction ($p \leq 0.05$) in the volume of paw edema in female adult Balb/c mice who received the nanoemulsion diet compared to the other experimental groups (control, formalin, fish oil and sunflower oil). These results indicate that the fish oil nanoemulsion was significantly effective in the dietary conditions tested here, presenting satisfactory responses in the modulation of inflammatory disorders, demonstrating interesting and beneficial nutraceutical effects.

1. Introduction

In the inflammatory process, ω-3 and ω-6 fatty acids act in the modulation of inflammatory reactions, producing biologically active mediators called eicosanoids with different stereochemistry [1–6]. The most important types of omega-3 are fatty acids, such as *cis*-4,7,10,13,16,19 docosahexaenoic (DHA) and *cis*-5,8,11,14,17 eicosapentaenoic (EPA), which usually produce mediators of low inflammatory potential. On the other hand, omega-6 fatty acids, such as arachidonic acid (AA), are used as a substrate to produce high-potential inflammatory eicosanoids, such as series-2 prostaglandins (PGE_2) and series-4 leukotrienes (LTB4) [7,8].

When an injury occurs in the cell membrane, inflammation begins after the production and release of these fatty acids that are produced

Abbreviations: 5-LOX, 5-lipooxygenase; AA, (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid (arachidonic acid); BBS, babassu; COX, cyclooxygenase; DHA, *cis*-4,7,10,13,16,19 docosahexaenoic acid; DLS, Dynamic Light Scattering; EPA, *cis*-5,8,11,14,17 eicosapentaenoic acid; FM, formalin; FO, fish oil; HPLC, High Performance Liquid Chromatography; LOX, lipooxygenase; LPS, gram negative bacteria lipopolysaccharide; LTB4, series-4 leukotrienes; MPO, myeloperoxidase enzyme; NaCl, sodium chloride; NE-blank, white nanoemulsion; NEW3, fish oil nanoemulsions; PGE_2, series 2 prostaglandins; PGE_3, series 3 prostaglandins; PUFAs, polyunsaturated fatty acids; RTCA, Real-time cell analysis; SEM, Transmission Scanning Electron Microscopy; SO, Sunflower oil; TAG, triacylglycerols; TXA3, thromboxanes; XDIR, 1,1'-dioctadeciltetramethyl indotricarbocyanine dye.

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from the membrane phospholipids, and the initial events of the inflammation occur through the AA cascade. In this metabolic pathway, oxidation of AA is initiated by the synthesis of lipid mediators. This process occurs through different mechanisms in the pathways: cyclooxygenase (COX), 5-lipooxygenase (5-LOX) (or 15-lipooxygenase and 12-lipooxygenase) and cytochrome P450. [6,9–13].

AA and EPA compete for the same receptors in the enzymatic pathways of COX and LOX. When EPA is used as substrate, it is possible to observe a decreased production of eicosanoids with high inflammatory potential. Thus, contributing to the synthesis of eicosanoids with low inflammatory potential of series 3 and 5 (odd), such as; prostaglandins (PGE_3), leukotrienes (LTB5) and thromboxanes (TXA3) [1,10,14–17]. By simultaneous action on the COX and LOX pathways, EPA and DHA are involved in the production of E series resolvins (from EPA) and D series resolvins, protectins, and maresins (from DHA), which are mediators that have strong anti-inflammatory potential acting in the resolution of inflammation [1,10,14–17].

The first key point of this work is that mammals are not able to naturally synthesize omega-3 polyunsaturated fatty acids, such as EPA and DHA, and eventually these acids must be obtained from the diet, mainly from some natural source rich in these fatty acids, such as fish oil (FO). The direct relationship of the anti-inflammatory effect of fish oil is strictly related to the mechanism of action of DHA and EPA, as presented in the previous paragraphs.

The second key point that will be addressed in this article is that most nutritional products produced from fish oil are highly hydrophobic products that, according to the literature [7,18], are poorly absorbed orally due to their limitation of low solubility in water. The oral bioavailability of DHA and EPA fatty acids in FO sources is strongly linked to solubilization in the gastrointestinal tract, and their lipid structure can influence their bioavailability [18].

To circumvent this disadvantage, our research group has been involved in projects with Nanotechnology to improve absorption and reduce the gastric emptying time of this type of lipophilic compound [19]. The idea is to disperse lipophilic compounds, such as FO, in stable nanodroplets. In this way, the loaded components can easily cross the mucous barrier, which is the main obstacle for lipid molecules, and reach the cell layer of the membrane to be absorbed [20]. Gastrointestinal mucus is a complex system that also acts as a selective physical barrier, allowing the rapid passage of small molecules [20,21]. Thus, the forms of delivery of a formulation must be designed with functional properties to adhere to the mucous membranes, allowing better absorption.

In this context, we designed the development of a nanoemulsified system for the release of lipophilic compounds, such as FO. Our main objective was to test whether the oral administration of a fish oil lipid nanoemulsion could improve the absorption of FO in experimental models *in vivo*, thus increasing the availability of anti-inflammatory omega-3 fatty acids. Thus, our hypothesis was that, based on the availability of DHA and EPA in the nanoformulated diet, oral supplementation with fish oil nanoemulsion (NEW3) could modulate the inflammatory response in experimental animals compared to animals treated with conventional FO.

2. Materials and methods

2.1. Methodological steps

To answer the hypotheses of this investigation, the following steps were planned in this study: (1) development and characterization of the colloidal stability of the NEW3 nanoemulsion; (2) evaluation of cytotoxicity and kinetics of *in vitro* incorporation of NEW3 nanoemulsion in mammalian cells; (3) *in vivo* evaluation of gastric emptying time of NEW3 nanoemulsion and free fish oil; (4) *in vivo* evaluation of the anti-inflammatory effect of the NEW3 oral diet (supplementation) in two experimental models.

2.2. Materials and reagents

Omega 3 fish oil capsules (Nu3 Health DHA-500) containing a mixture of DHA and EPA (DHA 0.5 g; EPA 0.1 g) obtained commercially from Natue Trade in Food Products, Ltda and natural oil from babassu (BBS) obtained from a local shop were tested in the oil phase nanoemulsion. Milli-Q ultrapure water was used as a vehicle in the formulation and the Solutol HS 15-polyoxyethylene esters of 12-hydroxystearic acid (Sigma-Aldrich, USA) used as an emulsifier.

Commercially available sunflower oil (composed of 0.58 g of polyunsaturated fats/48–74 % linoleic acid), 1 mg/mL of lipopolysaccharides (LPS), luminol (30 mg/mL), formalin (2.5 %) and sodium chloride (NaCl) (0.9 %) were used to study experimental *in vivo* models of inflammation. Other reagents were needed in the course of the experiments: all-*cis*-4,7,10,13,16,19-Docosahexaenoic acid methyl ester standard, 99.9 % purity, (Sigma-Aldrich, USA), HPLC grade methanol, Phosphate-saline buffer (PBS), DMEM (Dulbecco's Modified Eagle Medium, 1,1′-dioctadecyltetramethyl indotricarbocyanine iodide -XDIR (Caliper Life Sciences, USA), 1%.

2.3. Development of fish oil nanoemulsion (NEW3)

The nanoformulations were developed using the Phase Inversion Temperature method available and recommended in the literature [22,23] with adaptations. Fish oil was formulated considering the concentration of DHA and EPA; these concentrations of DHA and EPA as well as the other components used in the formulations were per mg/mL of emulsion and are available in Table 1.

To obtain the final formulation of fish oil (NEW3), screening was carried out based on the production of six nanoemulsions (Nano A, Nano B, Nano C, Nano D, Nano E and Nano F) (Table 1). At this stage, the Hydrodynamic Diameter (nm) and the Polydispersion Index (PDI) were evaluated over time, at 01 (after 24 h) and 30 days (**Supplementary Fig. S1**), and visual observations of the macroscopic characteristics, namely phase separation and color change, were performed.

The oily phases were tested, using fish oil and babassu oil (BBS). The aqueous phase was composed of Milli-Q Ultrapure Water (18 MΩ.cm), and the emulsifier used was Solutol HS 15-polyoxyethylene esters of 12-hydroxystearic acid (Sigma-Aldrich, USA). These components were chosen because of the ease in forming nanoemulsions with low energy demand.

A blank nanoemulsion (NE-blank) without fish oil from the final formulation was also prepared, following the same experimental procedures for comparison purposes in cell culture experiments. For the study of the characterization and colloidal stability, NEW3 samples were protected from light radiation and stored under two temperature conditions, 4 °C and 25 °C.

For *in vitro* tests using mammalian cells (RAW 264.7 macrophages) and *in vivo* tests in mice, the nanoemulsion was filtered using a sterile filter, coated with a 0.22 μm pore size membrane, non-pyrogenic (Kasvi, K18−230). Subsequently, the size was measured by Dynamic Light

Table 1
Nanoformulations of Fish Oil (FO) and concentrations (mg/mL) of the components.

Nanoemulsions Components (mg/mL)	Nano-A	Nano-B	Nano-C	Nano-D	Nano-E	Nano-F
DHA	6.25	3.125	6.25	3.125	1.5625	1.5625
EPA	1.25	0.625	1.25	0.625	0.3125	0.3125
Solutol HS15	18	18	32.5	32.5	18	20
Castor Oil	–	–	–	–	–	10
BBS	5	5	5	5	5	5

DHA: *cis*-4,7,10,13,16,19 docosahexaenoic acid; EPA: *cis*-5,8,11,14,17 eicosapentaenoic acid; BBS: Babassu oil; d =1 g/cm^3 para 20 g de H_2O q.s.p. Author search data (2017).

Scattering (DLS) (**Supplementary Fig. S2**). In *in vivo* tests, the therapeutic concentration tested was 50 mg/Kg DHA and 10 mg/Kg EPA per animal/day according to the recommendation of global oral intake [24–27].

2.4. Colloidal characterization and stability

2.4.1. Droplet size analysis

Droplet size and PDI were analyzed at different times (1, 7, 15, 30, 60, 180 and 338 days) after nanoemulsion preparation. The nanoemulsions were evaluated at two temperatures, 4 °C and 25 °C. The samples were diluted 1:20 (v/v) in Milli-Q ultrapure water (18 MΩ.cm) and measured by Dynamic Light Scattering (DLS) [28,29] using Zetasizer Nano (ZEN3690 model, Malvern Instruments, United Kingdom).

2.4.2. Determination of zeta potential

The zeta potential was measured after 1, 7, 15, 30, 60 and 180 days and evaluated at two temperatures, 4 °C and 25 °C. The formulation was diluted 1:20 (v/v) in ultrapure water Milli-Q (18 MΩ.cm) and analyzed using Zetasizer Nano (model ZEN3690, Malvern Instruments, United Kingdom). Smoluchowski's model was used to calculate the zeta potential of the systems [30].

2.4.3. pH measurement

The pH values of nanoemulsions were measured after 01, 07, 15, 30, 60 and 180 days at two temperatures, 4 °C and 25 °C. A pH meter was used, accuracy ± 0.01, sensitivity 100 % (NTPHM model, New Technology, Brazil), calibrated with standard solutions at pH 4.0 and pH 7.0, and the samples were diluted 1:10 (v/v) in Milli-Q ultrapure water (18 MΩ.cm) [31,32].

2.4.4. Scanning electron microscopy – SEM

Visualization of morphology of fish oil nanoemulsion was carried out by Transmission Scanning Electron Microscopy (SEM). An Electron Scanning Microscope (Jeol, JSM-7000 F, US) coupled to an EDS system (NSS 2.2 X-Ray MicroAnalysis, Thermo Scientific, US) was used. The samples were diluted 1:500 (v/v) in Milli-Q ultrapure water, and 100 μL of the dilution was deposited on a smooth and polished iron stub surface. The samples were contrasted with 2% OsO_4 vapor for 30 min to label the lipids and left overnight to dry samples at room temperature. After complete drop drying, nanoemulsion was metallized with Platinum film on a Sputter Coater (Balzers SCD 50, USA). The images were collected at an increase of 10,000 x with 15.0 kv.

2.4.5. Preparation of the DHA calibration curve and quality control

To obtain the DHA standard calibration curve, triplicate solutions of the all-*cis*-4,7,10,13,16,19 docosahexaenoic acid methyl ester standard, 99.9 % purity, (Sigma-Aldrich, USA) in concentrations of (0; 0.01; 0.025; 0.04; 0.055 mg/mL) diluted in HPLC grade methanol were prepared for the standard curve (**Supplementary Fig. S3**). High performance liquid chromatography (HPLC) was used, with a chromatograph (Shimadzu-Prominence series 20 Model) coupled to a degasser (DGU 20A5), automatic sampler (SIL-20AHT), pump (LC-20AT), column oven (CTO-20A) and CBM- Controller 20A. The detector used was UV–vis (SPD-20A) and a wavelength adjusted to 210 nm.

The stationary phase consisted of a Kinetex C18 column (25cm × 0.4cm/5 μm of particle diameter) (Phenomenex), oven temperature of 30 °C. The mobile phase consisted of 88 % HPLC grade methanol; the analysis was performed with a flow of 1 mL/min, isocratic method, sample injection volume of 20 μL and 25 min. Data processing was performed using the LC Solution V 1.24 software (Shimadzu, Tokyo-Japan).

The quality control samples of nanoemulsion (NEW3) and fish oil (FO) were methylated in basic catalysis using methods adapted from ISO 5509 International Organization for Standardization [33] and Milinsk et al. [34] for obtaining the methyl esters of docosahexaenoic acid. The quantification of DHA (mg/mL) in the test samples was analyzed by HPLC adapted from Kotani et al. [35] and Salm et al. [36] in methanol and water (88:12 v/v) to confirm the presence of DHA. The concentrations of the methylated test samples were 0.001998 mg/mL for the NEW3 nanoemulsion and 0.03798 mg/mL for fish oil.

2.4.6. Raman measures

Raman microspectroscopy analyses were performed using LabRAM HR Evolution (HORIBA Scientific, Palaiseau, France) equipped with an 1800 lines/mm grating and CCD detector. Measurements were conducted with a 532 nm laser excitation source (10 mW) focused on the samples by an OLYMPUS microscope with a 50× objective.

2.5. In vitro cell experiments

2.5.1. Cell culture

The RAW 264.7 adherent murine macrophage lineage, immortalized from the ATCC American Type Culture Collection, Rockville, MD, USA, was used for this study. The cells were thawed at room temperature and expanded in 250 mL cell culture flasks, with sterile filter and 75 cm^2 growth area (Greiner bio-one, São Paulo, BR) in sterile DMEM medium (Gibco® Life Technologies, Ltd, USA), buffered with sodium bicarbonate ($NaHCO_3$), pH 7.2, supplemented with 10 % (v/v) Bovine Fetal Serum (SFB) (Gibco® Invitrogen ™, USA) and 1% (v/v) of Pen Strep antibiotic solution (Gibco® Invitrogen ™, USA) composed of 100 IU/mL penicillin + 100 μg/mL streptomycin. The cells were cultured and kept in an incubator oven at a temperature of 37 °C, an atmosphere of 95 % humidified air and 5% CO_2 until the formation of the cell monolayer reaches the confluence. The cells were detached from the flask surfaces by scraping using a squeegee of sterile polyethylene cells, non-pyrogenic and non-cytotoxic, and then centrifuged at 178.88 *g* RCF for 3 min, followed by resuspension in DMEM medium.

2.5.2. Real-time cell analysis (RTCA) of cytotoxicity

The real-time effects of NEW3 nanoemulsion and blank nanoemulsion (NE-blank) on murine macrophage cells (RAW 264.7) were monitored using the RTCA-DP system (xCELLigence, Roche Applied Science). The cells were grown in cell culture flasks until they reached 80 % confluence and then were seeded at 2.5×10^3 cells/well in two 16-well plates (E-Plate16) containing electronic biosensors to measure impedance generated by cell adhesion.

The RTCA experiment consisted of 3 main steps: (1) 50 μL of DMEM culture medium (at room temperature) was added to all wells and maintained for 30 min for calibration and verification of the correct electrode function; (2) 50 μL of cells were added to the wells to wait for diffusion scattering, and the plates were incubated for 24 h before treatment; (3) Then, the culture medium of each well was carefully discarded with the aid of calibrated micropipettes in the laminar flow cabinet; after disposal, the cells were treated with 150 u L of fresh medium containing the test samples, the plates were returned to the oven at 37 °C with 5% CO_2, and monitoring was started and maintained for up to 72 h.

The mapping of treatments with RAW 264.7 cells were: T1-NEW3: 0.048 mg/mL; T2-NEW3: 0.097 mg/mL; T3-NEW3: 0.120 mg/mL; T4-NE-blank: 0.048 mg/mL; T5-NE-blank: 0.097 mg/mL; T6-NE-blank: 0.120 mg/mL. Each experimental sample was analyzed in triplicate. The untreated RAW 264.7 cells served as a negative control.

2.5.3. Kinetics of DHA incorporation into macrophages

To evaluate the kinetics of DHA incorporation in RAW 264.7 macrophage cells the following steps were performed: a) plating at a concentration of 5.0×10^3 cells/well in two 24-well plates; b) cell growth for 24 h; c) treatment with fish oil nanoemulsion samples (NEW3) in concentrations of 0.024 mg/mL and 0.048 mg/mL; finally, the control consisted of untreated cells. The samples were monitored over time; 17, 18 and 19 h; d) Then, extraction of free DHA from the

monolayer of RAW 264.7 cells was performed with isopropyl alcohol P. A (Sigma-Aldrich, USA).

The DHA (mg/L) extraction step occurred as follows: I) the medium was removed from each well and 1 mL of P.A. isopropyl alcohol (Sigma-Aldrich, USA) was added. The system remained in an environment saturated with the isopropyl alcohol solvent P.A overnight; after that, the solvent was collected from each well and evaporated with N2 vapor. II) after evaporation, the samples were resuspended in 1 mL of HPLC grade methanol and transferred to previously identified Eppendorf tubes and centrifuged for 10 min at 2236 *g* RCF. III) the supernatant was collected and measured using the HPLC technique. The samples were analyzed in triplicate (n = 3), and the operational parameters are similar to those described in item 2.4.5.

2.6. In vivo experiments

To answer the question of whether oral administration of a fish oil nanoemulsion (NEW3) reduces gastric emptying time and, consequently, improves the absorption of FO, thereby increasing the availability of anti-inflammatory omega-3 fatty acids in membrane phospholipids, thus mediating inflammatory processes, we applied studies to experimental models *in vivo*. First, we observed the gastric emptying time in two independent experiments and second, we analyzed the effect of NEW3 in two inflammatory models.

2.6.1. Ethics statement

This study was approved by the Animal Use Ethics Committee (CEUA) of the University of Brasilia under protocol no. 7/2017 (CEUA-UnB-7/2017) and respects the National Ethical Standards in accordance with Federal Law 11.794 of October 08, 2008 [37], and the resolutions of the National Council for Control of Animal Experimentation −CONCEA (BR) [38], and other international standards applicable to the use of animals for teaching and research, in particular the UK Animal Law (Scientific Procedures) 1986 and associated guidelines [39], ARRIVE guidelines.

2.6.2. Animal model

The animal model used consisted of healthy adult female isogenic mice, *Mus musculus*, Balb/c, aged between 10 and 12 weeks and with a body mass of approximately 16.0–31.0 g. The animals were supplied by the central vivarium of the Pontifical Catholic University of Brasilia (Brazil) and kept in the vivarium of the Department of Genetics and Morphology (UnB, Brazil) in polypropylene cages (30 × 20 × 13 cm) covered with stainless steel ASI wire. The cages were cleaned and sanitized three times a week and were kept in a low-speed, continuous airflow system, with excellent air exchange and internal pressure balance. The mice were kept at a controlled temperature (22 °C ± 2 °C) with a 12/12 -h light/dark cycle, with *ad libitum* water supply and controlled purine pelleted feed.

2.6.3. In vivo experimental groups

For the gastric emptying time model, three (3) experimental groups were tested: (I): XDIR, (II): fish oil (FO) and (III): NEW3, with n = 5 animals/group. For the first model of Inflammation, namely Sepsis induced with bacterial lipopolysaccharides (LPS), four (4) experimental groups were tested; (I): PBS control, (II): NEW3, (III): fish oil (FO) and (IV): sunflower oil (SO), each group with n = 5 animals. For the second model of inflammation, which was Paw Edema induced with Formalin, five (5) experimental groups were tested; (I): PBS control (Saline), (II): Formalin (FM), (III): sunflower oil (SO), (IV): fish oil (FO) and (V): NEW3, all with n = 7 animals/group.

2.6.4. Oral diet - treatments (doses)

For each experimental group of the gastric emptying test, 200 μL of the sample diluted in sterile PBS was administered orally (gavage) corresponding to a single dose for each treatment: group (I) XDIR with 57.36 mg/Kg of XDIR; group (II) Fish oil (FO) with 100 mg/Kg of FO (containing 50 mg/Kg of DHA and 10 mg/Kg of EPA) and group (III) nanoemulsion of fish oil (NEW3) with 100 mg/Kg of NEW3 (containing 50 mg/Kg of DHA and 10 mg/Kg of EPA). These doses were administered before the visualization procedures using the *in vivo* imaging technique. The group of labeled fish oil (FO) comprised the control sample.

For the two experimental models of Inflammation (Sepsis and Paw Edema) the groups NEW3, FO and SO received a daily dose of 100 mg/Kg of NEW3 (containing 50 mg/Kg of DHA and 10 mg/Kg EPA), 100 mg/Kg of FO (containing 50 mg/Kg of DHA and 10 mg/Kg EPA) and 100 mg/Kg of SO (containing 58 mg/Kg of polyunsaturated fats/higher % linoleic acid), respectively.

It is important to highlight that for these models the daily doses of the diet with source of omega 3 fatty acids were based on the concentrations of DHA 6.25 mg/mL and EPA 1.25 mg/mL contained in 12.5 mg/mL of oil of fish, and source of omega 6 in the concentration of 7.30 mg/mL of polyunsaturated fats/higher% of linoleic acid contained in 12.5 mg/mL of sunflower oil. These concentrations were diluted in sterile PBS and administered orally by the gavage method for 21 days. The animals were supplemented daily, at the same time at 2 pm, with each sample according to the experimental group.

The time of administration and doses applied were established based on global recommendations for the intake and supplementation of sources of polyunsaturated fatty acids and studies of the composition of these acids in mammalian tissues [24–27]. Before performing the treatments on the inflammation models, the gastric emptying time experiment of the NEW3 nanoemulsion and fish oil (FO) was carried out according to the method described below.

2.6.5. Stomach emptying time

This experimental protocol evaluated the emptying time of the stomach for the NEW3 and fish oil (FO) samples, with fasting animals (12 h) and fed animals (two independent experiments with an interval of one week between tests) with free access to water (*ad libitum*) and controlled feed.

The samples of each treatment corresponding to the experimental groups (I): XDIR, (II): fish oil (FO) and (III): NEW3 with n = 5 animals/group were marked with a fluorescent lipophilic 1,1′-dioctadeciltetramethyl indotricarbocyanine dye (XenoLight DiR) (Caliper Life Sciences, USA) in the concentration of 7.17 mg/mL, 1% (w/w) of the total lipids, referred to in the present work as XDIR.

Before the collection of images, the animals were treated with a single dose of 200 μL of the corresponding sample per experimental group. To collect the images, the experimental mice were anesthetized with isoflurane gas for 30 s and observed with fluorescence images *in vivo* IVIS Lumina XR, Version 4.3.1 (Caliper Life Sciences, USA). Quantification was collected 0.5, 2, 4, 6 and 24 h after application of the samples. The acquisition parameters were: excitation and emission at 720 and 790 nm, respectively; Field of view (FOV): C, Subject height (SH): 1.50 cm, Image mode: fluorescent photography, image (photography) p = 2–8, high lamp level and the photons quantified in average radiance [p/s/cm^2/sr].

2.6.6. LPS-induced sepsis model

Sepsis induction was performed with LPS - gram negative bacteria lipopolysaccharide (Merck Millipore), for which the animals received a 50 μL subcutaneous dorsal injection at a dose of 2 mg/Kg LPS (1 mg/mL). At 2 h and 24 h after the inflammatory challenge, the animals were evaluated for the expression of the myeloperoxidase enzyme (MPO) using the *in vivo* bioluminescence technique. This enzyme is predominantly expressed in neutrophils [40], and the inflammatory evaluation in this model was related to neutrophil infiltration. The survival profile up to 48 h after induction was also assessed.

For imaging, the animals were anesthetized with isoflurane (1.5–2 %) gas for 30 s and received a 250 μL intraperitoneal injection at the

dose 300 mg/Kg of luminol (30 mg/mL), which is sensitive to myeloperoxidase (MPO) activity [41]. The data *in vivo* were quantified by extension of the photons generated in average radiance [p/s/cm^2/sr] and acquired through IVIS Lumina XR, Version 4.3.1 (Caliper Life Sciences, USA). The operating parameters were: Figure Exposure: 60 s; Sequence: 30 photos/1 min; Binning: 8; F/Stop: 1; Excitation Filter: block; Emission Filter: open and field of view (FOV): D, Subject Height (SH): 1.50 cm, Imaging Mode: luminescent photography, image (photography) p = 2–8, Focus: use subject height.

2.6.7. Formalin-induced inflammation model paw edema

For the second challenge of experimental inflammation, a sodium chloride saline solution (NaCl) (0.9 %) was prepared and applied to the Control (Saline) group, and this solution constituted the negative control. A Formalin solution (2.5 %) was prepared and applied to the group FM (Formalin) as a positive control. In these two groups, PBS was administered during the supplementation period. The treated groups: SO (sunflower oil), FO (fish oil) and NEW3 were tested, and the paw edema model was induced with formalin. Before induction, the animals were anesthetized with an intraperitoneal injection of 100 µL of a mixture of 10 % Xylazine Hydrochloride and 10 % Ketamine Hydrochloride in distilled water; after anesthesia, the basal measurement of the right and left paws was performed with a paw volume meter, Pletysometer (Bonther, Brazil).

Then, edema was induced in the mouse's right hind leg with a subplantar (subcutaneous) injection of 50 µL of formalin (2.5 %) [42] in the groups: FM, SO, FO and NEW3. The saline control group received a subplantar injection of NaCl (0.9 %). The volume of the paw of the mice was measured three times on the plethysmometer at times: 0, 15, 30, 45 and 60 min. The percentage of increase in the volume of paw edema was calculated based on the formula:

$$Paw\ volume\ (\%\ increase) = \frac{Va - Vb}{Vb}\ x\ 100$$

Where *Va* is the average paw volume at specific time intervals and *Vb* is the average paw volume before formalin injection.

2.6.8. Quantification of prostaglandins E_2 (PGE_2) in tissue

The right paws with edema were collected to quantify the concentration of prostaglandins PGE_2. For the extraction of prostaglandins, the subplantar region of the paw was collected, weighed and transferred to 1.5 mL microtubes, previously weighed. Then 1 mL of the 0.1 M homogenization buffer of anhydrous monobasic potassium phosphate, pH 7.4, containing 1 mM EDTA and 10 µM indomethacin was added per gram of tissue to the tubes.

Then, the tubes were homogenized and centrifuged at 8000 *g* of RCF, at 18 °C for 10 min. The supernatant was collected and the concentration (pg/mL) of the PGE_2 analyte was performed by means of a competitive immunoassay test with labeled PGE_2 antibody. The assay is based on the competition between the PGE_2 analyte contained in the standard or on sample between the PGE_2 conjugated to the Alkaline Phosphatase (PGE_2-AP) with an amount of mouse monoclonal antibody specific for PGE_2. The plate is pre-coated with an anti-mouse IgG antibody. All assay procedures were in accordance with the instruction manual available in the PGE_2 ELISA KIT (Invitrogen, Thermo Fisher Scientific, Austria).

2.6.9. Hematological and tissue testing

An aliquot of the blood of the mice from the experimental groups of the paw edema model was collected to quantify the hematological indexes. The blood was transferred to tubes with anticoagulant and the analyses were measured on the hematimeter (LabTest, Brazil), where the following parameters were read: Erythrogram [RBC = total red blood cell count (x10^6/µL), HGB = hemoglobin concentration (g/dL), HCT = hematocrit percentage (%) and PTL = platelets count (x10^3/µL)]. Leukogram [WBC = total white blood cell count (x10^3/µL), W-SCC = absolute small white blood cell count - lymphocytes (x10^3/µL); W-MCC = intermediate cell count - monocytes (x10^3/µL) and W-LCC = large cell count - neutrophils (x10^3/µL)].

2.6.10. Clinical essays

During the period of supplementation with experimental treatments in the two models of inflammation, the feed was controlled and supplied with a specific amount of 200 g per group. Every seven days, the resulting feed mass was collected and weighed per group, and again another feed batch was replaced. The animals were also weighed individually every seven days and were evaluated for clinical aspects: body mass (g) and individual mass gain (g/d) calculated using the formula:

$$\text{individual mass gain}\ (\text{g/d}) = \frac{Mf - Mi}{T}$$

Where: *Mf* is the final average mass of the animal in grams (g); *Mi* is the average initial mass of the animal in grams; *T* is the supplementation time in days. The feed efficiency was determined by means of the feed efficiency coefficient (CEA), which is the relationship between the mass gained and the amount of food consumed [43], was calculated using the formula:

$$CEA\ (\text{g/d}) = \frac{MFs - MIs}{QTA}$$

Where: *MFs* is the animal's final mass after the experiment (supplementation) in grams (g), *MIs* is the animal's body mass at the beginning of the experiment (supplementation) in grams (g); *QTA* is the total amount of food consumed in the experiment period in grams (g). In addition to these parameters, visual observations of animal mobility and survival profile were also performed.

2.7. Statistical analysis

The results collected in the present study were evaluated considering the value of *p* with the significance level of $p \leq 0.05$ from the statistical analysis of multiple comparison, ANOVA. The *in vivo* statistical planning for the sample size (n) was calculated based on the formula regulated by the Animal Use Ethics Committee of the University of Brasília (CEUA/UnB), considering the power of the 90 % test and the level of significance $p \leq 0.05$. The mean standard deviations were plotted, in which values of $^*p \leq 0.05$ were considered statistically significant; the graphs were produced using the GraphPad version 6.0 biostatistics program (GraphPad, United States of America).

3. Results and discussion

3.1. NEW3 preparation

The final FO nanoemulsion was defined here as NEW3, due to the presence of Omega-3 in the sources of FO; it was established after evaluating screening and stability for one month of six different nanoemulsion preparations. The difference in each formulation was the oil/surfactant ratio. A final nanoemulsion chosen from Fish Oil (FO) was composed of FO (12.5 mg/mL; containing 6.25 mg/mL of DHA and 1.25 mg/mL EPA) and babassu oil (5.0 mg/mL) as an oil phase; and solutol (18.0 mg/mL) as a single surfactant for nanoemulsion.

Nano A was the only sample tested that showed no signs of macroscopic instability and remained within the range (mean size <200 nm and PDI < 0.3) after one month of observation at room temperature (**Supplementary Fig. S1**) in comparison with the other samples. The Nano F sample presented results within the range, but it showed signs of macroscopic instability, such as color change, at the end of the investigated time.

Therefore, Nano A was selected to be used as the test nanoemulsion for the experiments and named NEW3 (fish oil nanoemulsion). The

NEW3 nanoemulsion also underwent size validation tests and PDI after filtration (**Supplementary Fig. S2**). The purpose of filtration in syringe filters with 0.22 μm membranes was to make the system as sterile as possible for application in *in vitro* experiments and *in vivo*. In the validation we found that there was a reduction in the size of 2.85 % for the *in vitro* samples and 27.16 % for the *in vivo* samples; however, no significant differences were observed for the polydispersion index. This procedure also did not affect the identification of the DHA biomarker in the nanoemulsion (**Supplementary Fig. S3**).

3.2. Droplet size analysis and PDI

Fig. 1 shows the colloidal characterization of NEW3, for which we measured different aspects of NEW3 stability, firstly the macroscopic aspects were maintained during all periods analyzed without phase separation. In addition to macroscopic observation, we also measured the hydrodynamic diameter (Fig. 1A) and polydispersity index - PDI (Fig. 1B).

The NEW3 nanoemulsion showed size intervals at 4 °C in the range 161.455 ± 3.753 nm (1 st day) at 145.211 ± 6.617 nm (338 days) as a function of time, without significant changes ($p \leq 0.05$); and size range at 25 °C from 161,600 ± 3498 nm (1st day) to 162,933 ± 7967 nm (338 days). At this temperature, only a slight change in the significant size ($p \leq 0.05$) was observed at a single time, which was 180 days. The PDI values at 4 °C were in the range of 0.234 ± 0.013 (1st day) to 0.211 ± 0.034 (338 days). At the temperature of 25 °C, the range found was from

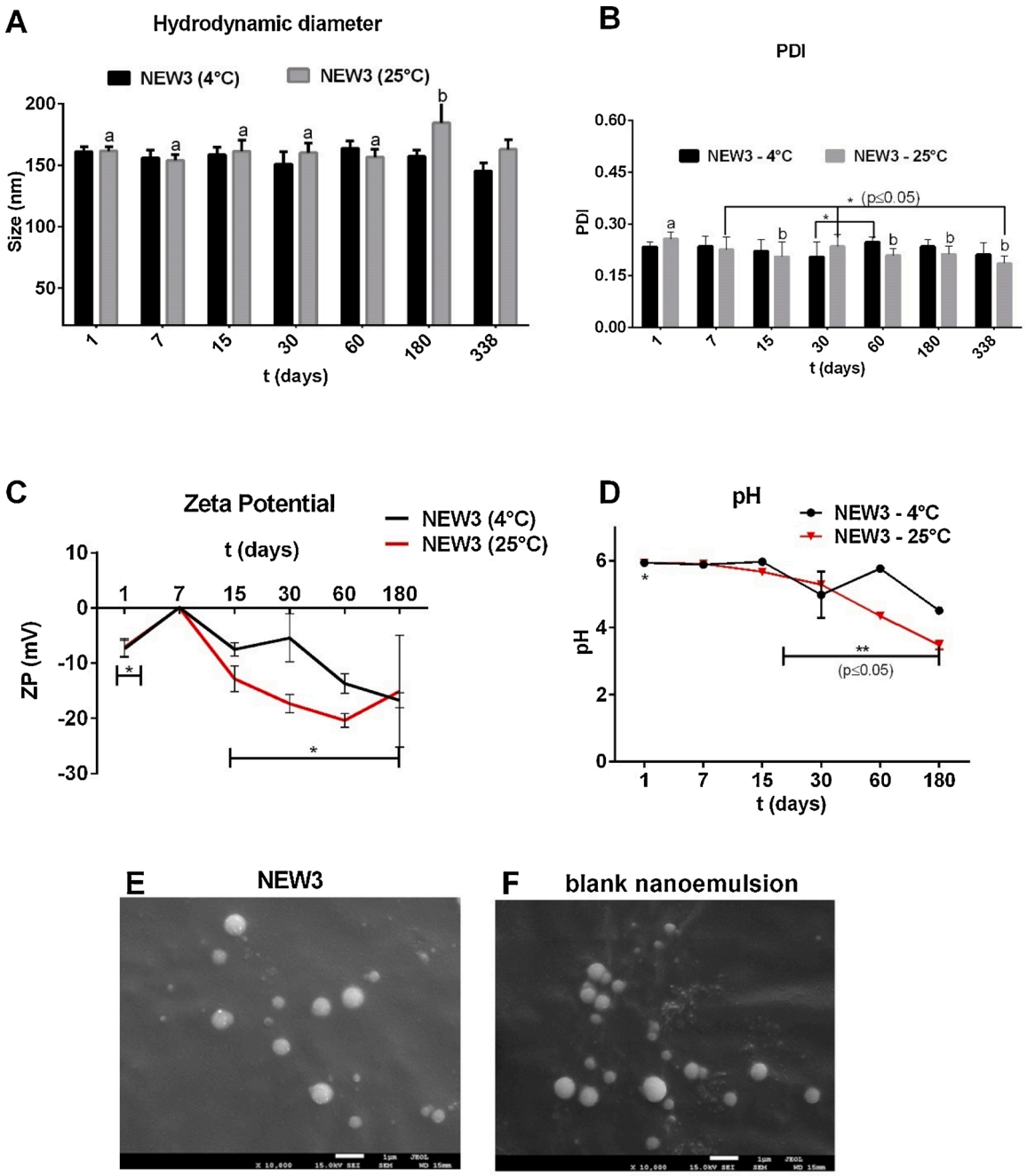

Fig. 1. Colloidal characterization of nanoformulations at 4 °C and 25 °C as a function of time. **(A)** Hydrodynamic diameter - DH (nm); **(B)** Polydispersity index - PDI; **(C)** Zeta potential (mV); **(D)** pH, **(E)** Scanning Electron Microscopy - SEM of the NEW3 nanoemulsion; **(F)** Scanning Electron Microscopy - SEM of blank nanoemulsion. **Legends**: **NEW3:** nanoemulsion with fish oil; t: time. Bar graphs with different letters and asterisks (*) show a significant difference ($p \leq 0.05$) when comparing the times between them, based on Tukey's multiple comparison test, 2way ANOVA multiple comparisons. The data are represented as mean ± SD, n = 3.

0.256 ± 0.019 (1st day) to 0.185 ± 0.021 (338 days), and it was noted that in both temperature conditions there was a decrease ($p \leq 0.05$) of initial value of the PDI, demonstrating that the nanoemulsion remained as a monodispersed system as a function of time with averages below 0.25.

The size results for NEW3 are in agreement with the results of Walker et al. [44], who studied the physical stability at 37 °C and fish oil oxidation when entrapped in nanoemulsions produced by spontaneous emulsification. The researchers produced nanoemulsions of fish oil mixed with lemon oil and found droplet size values in the range of 96.5 ± 2.0 nm to 166.5 ± 1.8 nm.

Another study of the properties and stability of fish oil nanoemulsions was carried out by Nejadmansouri et al. [45], who formulated fish oil nanoemulsions stabilized with different mixtures of non-ionic surfactants (Tween 80 and Span 80) and evaluated DH at two temperatures (4 °C and 25 °C) for 1 month. The authors found no significant differences ($p < 0.05$) in sizes at 4 °C as a function of the investigated time, finding the range from 100 to 200 nm; however, they were able to observe increasing size variations in temperature at 25 °C at 14 days with intervals of 400–500 nm.

Considering the development of new products based on this nanoformulation, the long-term colloidal stability of NEW3 (Fig. 1A, **1B**) is a very important discovery in our results, as the stability continued for one year, when evaluated at the end of 338 days under two different temperature conditions.

3.3. Zeta Potential, pH and SEM

The values of ZP at 4 °C ranged from -7.381 ± 1.528 mV (1 st day) to -16.744 ± 1.347 mV (180 days). At a temperature of 25 °C, the range found was -7.120 ± 1.616 mV (1 st day) to -15.094 ± 10.116 mV (180 days) (Fig. 1C). These intervals were already expected for this system. In the work of [44], the ZP values obtained were -1.5 mV (day 0) and -2.0 mV (14 days), relatively lower than those found in the present study; however, in both conditions, they are in agreement with the load of droplet surface showing negative and slightly increased over time.

ZP is an indicator of the stability of a colloidal suspension. Systems that have non-ionic surfactants with high molecular weight and absolute values of ZP around 20 mV (in module) or lower can provide sufficient stabilization, due to the chemical properties of these surfactants to act mainly by steric stabilization. The addition of non-ionic surfactants does not affect the surface potential [30].

The NEW3 nanoemulsion pH ranges found at 4 °C were 5.935 ± 0.053 (1 st day), and 4.508 ± 0.037 (180 days); for 25 °C they were 5.94 ± 0.055 (1 st day) and 3.48 ± 0.132 (180 days) (Fig. 1D). A study in the literature [46] with fish oil liposomes measured over 3 months showed mean pH equivalent to 4.53 ± 0.11, corroborating the results found in the present study.

Interestingly, both the dispersion pH and zeta potential measurements changed slightly depending on the observation time, but these changes did not significantly affect the integrity of DHA in the nanoemulsion, which was detectable for at least one year of observation (**Supplementary Fig. S3**), and the transported DHA was chemically protected by the structure of the nanoemulsion. These two measurements (zeta potential and pH) correlate with each other, and therefore the PZ is pH-dependent; so the control of the ZP is possible by controlling the pH.

NEW3 morphology was observed using Scanning Electron Microscopy (SEM). Fig. 1**E** and **F** show the morphological aspects of NEW3 and the blank nanoemulsion, respectively. Notably, the droplets present in the samples showed an appearance with a spherical pattern, the distribution of the relative frequency of the average size of the NEW3 nanoemulsion was 134.31 ± 28.50 nm (**Supplementary Fig. S4**).

Properties such as size, shape and surface charge of a particle play a fundamental role in cell absorption. Negatively charged spherical particles can potentially bind to cationic sites available on the surface of the macrophage. Furthermore, negatively charged nanoemulsions have a higher uptake liver macophages compared to neutral nanoemulsions or positively charged nanoemulsions [47]. After these first characterization results, we proceeded to the investigation of the quality control of the system and subsequent biological tests.

3.4. Quality control - Raman measures

DHA was used as a biomarker of fish oil (FO) and the concentration of this fatty acid was confirmed by HPLC analysis (**Supplementary Fig. S3**) in the nanoemulsion (NEW3) and FO samples. In this assay we used external standardization, where analytical curves were constructed by comparing the areas of the DHA fatty acid to be quantified in the NEW3 samples and the fish oil with the areas obtained from the solutions of known concentrations prepared from the methyl ester standard all-*cis*-4,7,10,13,16,19, docosahexaenoic.

The linearity of the method was determined from the mathematical relationship $y = ax + b$ between the measured signal and the concentration of DHA in the analytical curve. The linear determination coefficient (R2) was obtained to estimate the quality of the obtained curve. The linear correlation coefficient (r) measures the degree of association (covariation) between two variables and can be calculated using the equation $r = \pm\sqrt{R2}$ where the value (r) is ≥ -1 and ≤ +1. The closer the linear correlation coefficient (r) to +1.0, the smaller the dispersion of the set of experimental points and the lower the uncertainty of the estimated regression coefficients [48].

In our tests, for the analytical curve of the methyl ester standard all-*cis*-4,7,10,13,16,19-docosahexaenoic - DHA we found the value of (r) = 0.9999; for NEW3 the value of (r) = 1.0 and for fish oil the value of (r) = 1.0. For each analytical curve, a model adjustment was obtained of 99.98 % (standard), 100 % (NEW3) and 100 % (fish oil), thus helping to explain all the variability of the data around the averages of the samples. The quantification of the presence of DHA served as a quality parameter, as this biomarker was identified and maintained in the nanoemulsion. Therefore, we proceeded to assess the chemical stability of NEW3 using Raman measurements.

To investigate the chemical stability of NEW3 (25 °C) as a function of preparation time, Raman measurements at t = 0, 365 and 730 days were performed. These results are shown in Fig. 2. Raman spectra show several distinct peaks that are typical for lipids. A more detailed assignment of the relevant signals is given in reference [49]. In Fig. 2 **(a)** the main spectral differences are observed in the vibrational regions associated with carbon double-bonds $C = C$, which are associated with the degree of lipid unsaturation. Highly unsaturated fatty acids derived from fish oils, particularly EPA and DHA, are particularly important in modulating the inflammatory processes [50].

However, DHA and EPA molecules are susceptible to auto-oxidation, which begins by attacking polyunsaturated fatty acid (RH) by a species with sufficient reactivity to remove a hydrogen atom from a methylene group ($-CH_2-$) (adjacent to a double bond = CH) leading to the initiation of lipid oxidation. At this stage, there is the formation of a lipid radical centered on methylene carbon (- $^{\bullet}$CH-), which combines with O_2 to produce the peroxyl radical (ROO$^{\bullet}$), which is even more reactive. This radical removes a hydrogen atom from another unoxidized fatty acid molecule in a chain reaction ($ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$) and in this way propagates the oxidation reaction [50].

The structural occurrence of multiple double bonds in DHA and EPA in the presence of free radicals allows the generation of several oxygenated metabolites. The combination of the peroxyl radical (ROO$^{\bullet}$) with an abstracted hydrogen atom forms hydroperoxide (ROOH) as a primary product [50]. According to Derogis et al. [51], the oxidation of DHA leads to the formation of at least ten monohydroperoxide isomers (HpDHA) all containing conjugated dienes.

As results, when lipids are oxidized, *cis* double bonds of hydrophobic chains rearrange to form *trans* double bonds [52,53]. Due to this fact,

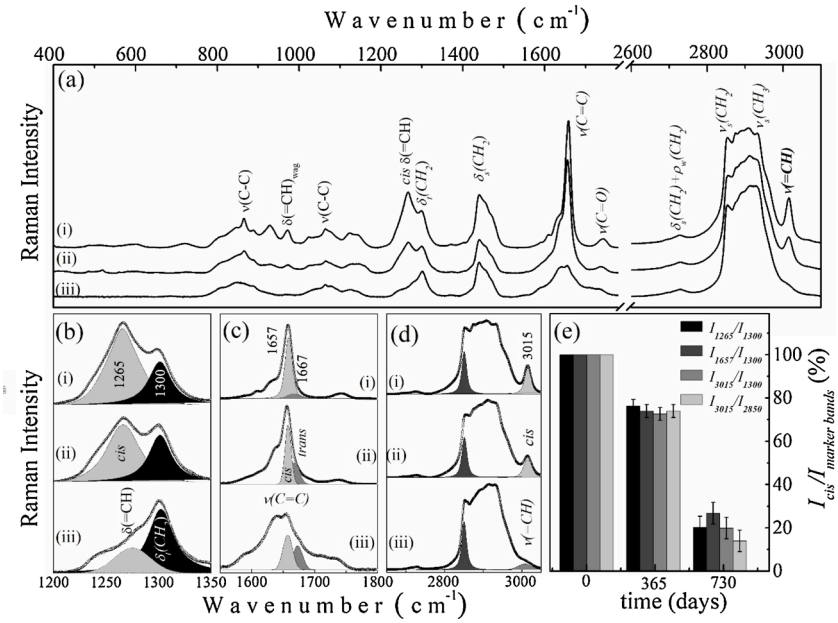

Fig. 2. Raman spectra of nanoemulsion NEW3 at 25 °C, performed after 0 (i), 365 (ii) and 730 (iii) days **(a)**. Zoom of spectral regions 1200 – 1350, 1550 – 1800 and 2650 – 3100 cm^{-1}, associated with δ_*cis* (=CH), δ_t (⟦CH⟧ _2); *cis*, trans ν(C═C) and ν(=CH), ν(⟦CH⟧ _2) vibrational modes, respectively **(b)**, **(c)** and **(d)**. The solid lines show the Lorentzian fits of the experimental data. Relative unsaturation (%) at time t = 0 days estimated from ratio between integrated intensities of bands at 1265/1300 cm^{-1} (black), 1657/1300 (dark gray), 3015/1300 (gray) and 3015/2850 cm^{-1} (light gray) **(e)**.

there is a decrease in the total degree of *cis* unsaturation and an increase in the levels of the *trans* unsaturation. Note that this behavior can be observed in Fig. 2 **(d)**, where the integrated intensity of the vibrational modes *cis* $\nu(C=C)$ (light gray - 1557 cm^{-1}) and *trans* $\nu(C=C)$ (dark gray - 1567 cm^{-1}) are highlighted. Therefore, the behavior shown in Fig. 2 **(d)** evidences that an oxidative process is present.

In order to assess the degree of oxidation of NEW3 samples over preparation time, the ratios between the integrated intensities of bands associated with *cis* double bonds and the integrated intensity of marker bands were taken, which do not change their intensity with the degree of oxidation. Sadeghi-jorabchi et al. [54] found that the ratio between the band around 1265 cm^{-1} (in-plane═C—H deformation of unconjugated *cis* double bonds - $\delta_{cis}(=CH)$) and the one around 1300 cm^{-1} (in-phase methylene twist deformation - $\delta_t(CH_2)$) is highly correlated to the *cis* isomer unsaturation.

A similar procedure can also be used for the $\nu_{cis}(C=C)$ and *cis* $\nu_{cis}(=CH)$ bands found around 1557 and 3015 cm^{-1}, respectively. In addition, Jamieson et al. [53] showed that the number of *cis* double bonds can also be obtained using the intensities ratio of stretching vibrations of the = CH and CH_2 bonds $\frac{(I_{3015})}{I_{2850})}$. The plots of $\frac{(I_{1265}}{I_{1300})}$, $\frac{(I_{1657}}{I_{1300})}$, $\frac{(I_{3015}}{I_{1300})}$ and $\frac{(I_{3015}}{I_{2850})}$ *versus* preparation time are shown in Fig. 2 **(e)**. Fig. 2 **(b)**, **(c)** and **(d)** show the zooms of spectral regions 1200–1350; 1550–1800, and 2650 – 3100 cm^{-1}, associated with $\delta_{cis}(=CH)$, $\delta_t(CH_2)$; *cis, trans* $\nu(C=C)$ and $\nu(=CH)$, $\nu(CH_2)$ vibrational modes, respectively.

These findings demonstrate that the NEW3 sample has a long-term chemical stability of up to 365 days; however, signals of initial chemical instability (~ 25 %) were measured after the first year of observation, reaching ~ 80 % of reduction at 730 days of observation. We consider that the presence in the formulation of babassu oil, a well-known antioxidant oil, may have contributed to the chemical stability of the nanocarrier.

This antioxidant property of babassu oil has already been investigated in our previous work with nanoemulsions, generating a patent application [55]. In addition, PUFAs in natural oils are preferably distributed in the TAG sn-2 position, and chemical randomization studies [56] demonstrate that DHA is more stable to oxidation when located in the sn-2 position compared to the sn-1 position. Our NEW3 fish oil formulation composed of a higher proportion of DHA may also have contributed to preserving the structure of the encapsulated DHA, as it showed a slower process of oxidation of fish oil up to 365 days.

3.5. *In vitro cell experiments*

3.5.1. *RTCA - real time cell analysis of macrophage cytotoxicity*

After the stability tests and verification of the integrity of the NEW3 nanoemulsion, the cytotoxicity test was performed in a culture of RAW 264.7 macrophages by analyzing the cell adhesion index (CI) in real time. Fig. 3**A** and **B** show the proliferation pattern of RAW 264.7 cells under different conditions, and the results show the monitoring of samples in real time in contact with the cells.

We selected a cell lineage with monocyte/macrophage morphology, because it is an important cell involved in the immune system and because it is widely used to assess the effects of DHA and EPA on cell function [57–59]. As shown in Fig. 3**A** and B, the NEW3 and NE-blank treatments (blank nanoemulsion) did not impair the proliferation of macrophage cells. There were no statistical differences in the cell indices for NEW3 and NE-blank compared to the control group, presenting good results in terms of cell biocompatibility at the tested concentrations.

Real-time cell index (CI) measurement is a fast-continuous monitoring process that has several advantages over traditional methods that provide data at a single point in time. This analysis is important because it shows a measurement of electrochemical signals directly proportional to the number of live cells adhered to without the use of dyes, thus allowing a direct and continuous measurement of cells under physiological conditions. There were no changes in the CI for the treatments tested here when we analyzed the AUC of NEW3 (Fig. 3A) and the AUC of NE-Blank (Fig. 3B), so we can verify that the treatments did not show significant differences compared to the control.

This approach is interesting because it demonstrates that NEW3 treatments at concentrations of 0.048 mg/mL; 0.097 mg/mL and 0.120 mg/mL were not cytotoxic in macrophage cells, thus presenting biocompatibility in the studied lineage. Thus, we carried on the study with the same lineage in the next assay.

3.5.2. *Kinetics of DHA incorporation into macrophages*

After cytotoxicity experiments, we evaluated the kinetics of incorporating DHA into the same macrophage lineage (RAW 264.7). As shown in Fig. 3C, DHA was incorporated by the RAW 264.7 cell in both NEW3 nanoemulsion treatments (0.024 mg/mL and 0.048 mg/mL). In addition, we observed significantly higher incorporation kinetics for the 0.024 mg/mL treatment. As expected, for the control group, DHA levels were undetectable.

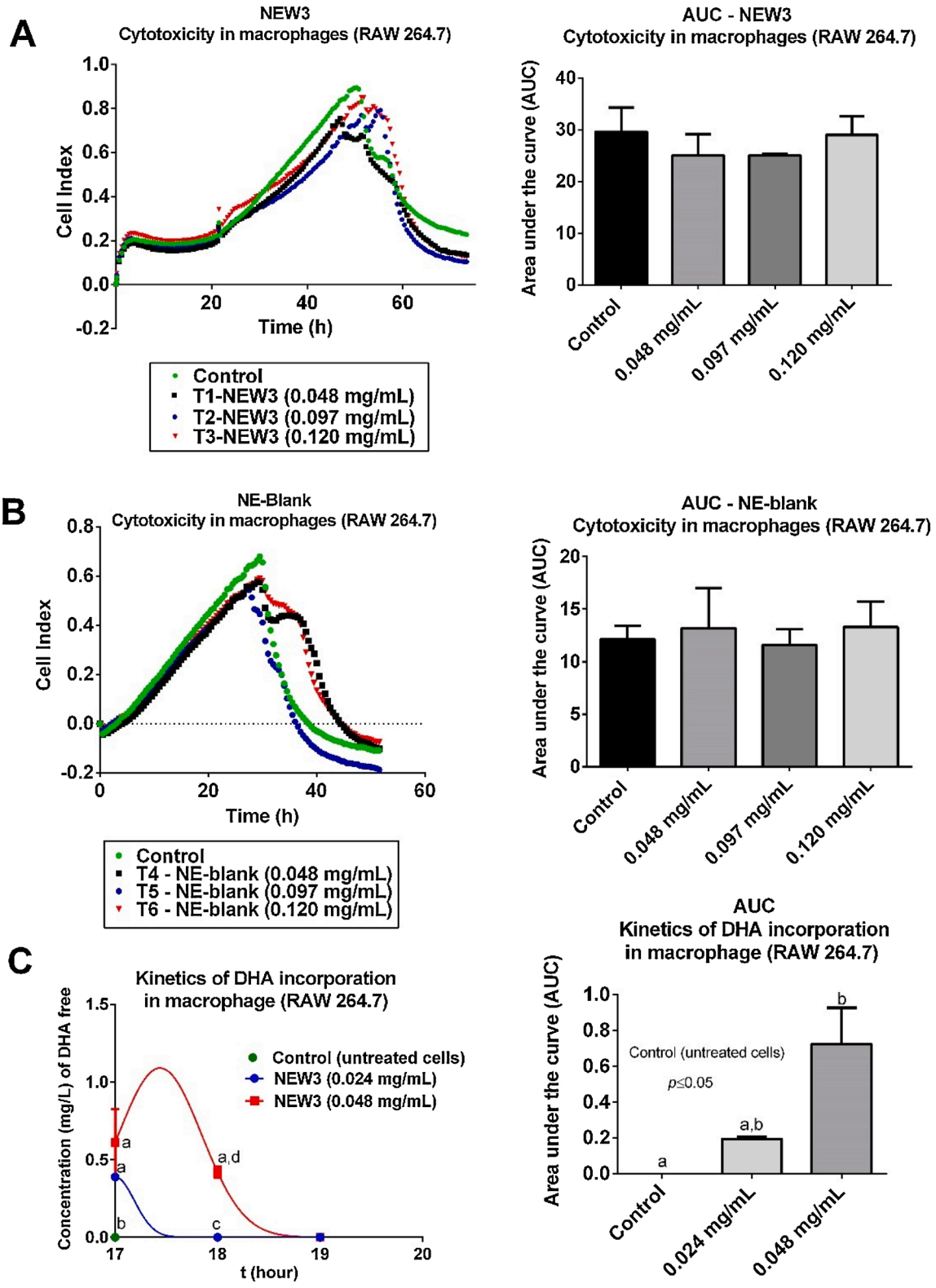

Fig. 3. *In vitro* experiments with RAW 264.7 macrophage cells. **(A)** Cell index in real time for NEW3 fish oil nanoemulsion; **(B)** Cell index in real time for NE-blank nanoemulsion; **(C)** Cell incorporation kinetics of *cis*-4,7,10,13,16,19 docosahexaenoic - DHA fatty acid. **Legends**: **NEW3**: fish oil nanoemulsion; **NE-blank**: white nanoemulsion without fish oil; **T(1… 6)**: treatments with samples in different concentrations; **t**: time. The data were expressed with the mean ± SD of three repetitions. The ANOVA multiple comparison test was applied with the level of significance $p \leq 0.05$. Different letters represent statistical differences *($p \leq 0.05$).

Fig. 3C demonstrates that the fatty acid DHA was effectively internalized by the cells in a concentration-dependent manner, as shown in the graph of the area under the curve. In this graph, the concentration of free DHA is clearly observed when the concentrations of 0.024 and 0.048 mg/mL of the nanoemulsion are compared. This is important evidence that demonstrates the ability of the NEW3 nanoemulsion to associate with biological membranes, a property that will be needed for *in vivo* absorption experiments.

The immunological modulating effects of polyunsaturated fatty acids (PUFAs) occur when these molecules are effectively incorporated into cell membrane phospholipids. Investigations of the kinetics of PUFA incorporation into cultured cells are models used to explore the absorption, metabolism and molecular effects of these fatty acids. To this end, the experimental conditions used for cell cultures are physiologically important in terms of the concentration of fatty acid in the culture medium.

Macrophages play a fundamental role in the onset of inflammation and are metabolically active, being distributed in the connective tissue that influences local and systemic functions [58]. The circulation of monocytes can vary after their production in the bone marrow [8]. In this interval of cell renewal, cells incorporate fatty acids (DHA and EPA) in their newly synthesized phospholipid membranes.

In the literature, studies demonstrate that diets with EPA and DHA fatty acids depending on the position in the dietary triacylglycerols (TAG) of fish oil may have an influence on their incorporation into cellular phospholipids and on the activity of phagocytic cells [60]. In the study by Adolph et al. [61], RAW 264.7 macrophage cells supplemented with docosahexaenoic acid (DHA) increase the rate of phagocytosis and the bactericidal capacity of macrophages. The authors support the idea of supplementing immunocompromised individuals with PUFAs, as well as people suffering from chronic infections.

It is important to note that these studies involve research on supplementation with omega-3 PUFAs based on concentrations of fatty acids without nanotechnological apparatus. In the present study, fish oil was associated in a nanoemulsion (NEW3) with a physiologically relevant 0.024 mg/mL (Fig. 3C) concentration, demonstrating kinetically the incorporation of DHA in less time in macrophage cells, so it is therefore a promising approach to modulating immune responses and attenuating the inflammatory signaling in *in vivo* models.

The absorption of nanoparticles by cells is a two-step process: first, a binding step on the cell membrane and then a second step of internalization. Therefore, the ability of nanostructures to adhere to biological membranes is one of the most desired objectives when thinking about nanocarriers for the transport of lipophilic compounds.

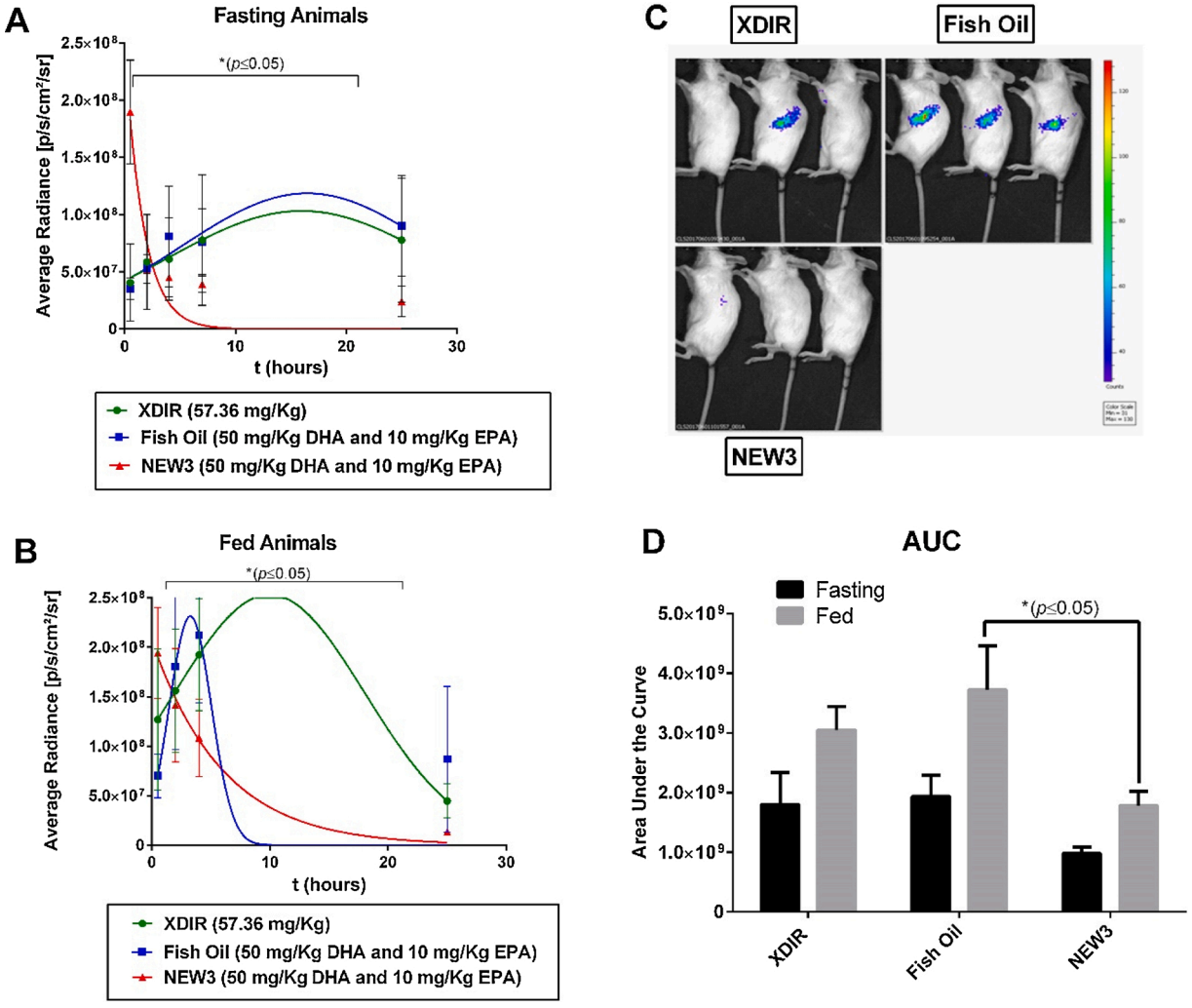

Fig. 4. Time of gastric emptying in two experimental models in the gastrointestinal system. **(A)**: animals fasting 12 h; **(B)** fed animals; **(C):** *in vivo* bioluminescence of the stomach region; **(D):** area under the curve of the experimental groups. **Legends**: **XDIR:** fluorescent lipophilic marker (1,1′-dioctadeciltetramethyl indotricarbocyanine iodide); **NEW3:** fish oil nanoemulsion; **SD:** Standard deviation. **t**: time, color scale: min. = 31, max. = 130. The data are plotted according to the mean ± SD of three repetitions. The bidirectional multiple comparison test - ANOVA was applied with the level of significance $*p \leq 0.05$.

3.6. In vivo experiments

3.6.1. Stomach emptying time

After experiments with the cytotoxicity tests and the incorporation of nanoemulsion in cells, we performed the first study in animals to evaluate the gastric emptying time of NEW3 in the gastrointestinal tract (TGI) in an *in vivo* imaging experiment. This experimental approach was previously published by our research group [19] with the aim of studying the emptying time of lipophilic molecules in the stomach, which can be correlated with the time it takes for samples to reach the intestine and be absorbed. The samples were marked with a sensitive fluorophore - XDIR.

In Fig. 4**A** it is possible to observe the results of the average radiance of the kinetics of the marked samples and of the fluorophore collected from the region of the animals' stomach for the different treatments in the fasting model. The graph shows that both treatments with fish oil (FO) and XDIR are kept in the stomach for longer periods, compared to NEW3, which has a faster gastric emptying time. In Fig. 4**B** the results of the kinetics of the samples marked for the model with the fed animals are shown, and again we found that the treatment with the NEW3 nanoemulsion showed similar characteristics to that observed in the fasting model, with a fast gastric emptying time. Important to highlight here that we are measuring the *in vivo* XDIR fluorochrome. The physical NE stability cannot be measured by this technique. However, based on the colloidal stability results, we can see that the hydrodynamic diameter (Fig. 1A) is kept stable for all time, even in conditions where the pH is reduced (Fig. 1D). So, based on this observation, we can suggest that the pH is not a determining variable for nanodroplet stability.

In Fig. 4C, there is a qualitative assessment of the bioluminescence of the experimental groups in the stomach region after 24 h. In this image, the retention of samples in the stomach region is evident for the groups XDIR and FO, while no fluorescent signal is observed for NEW3. These results demonstrate that necessarily the fish oil carried in NEW3 nanoemulsion was more advantageous for the oral biodistribution of this compound in the TGI.

It is interesting to note that when we analyzed the AUC of the groups (Fig. 4D), we observed that for both conditions, fasting and fed animals, NEW3 provides shorter gastric emptying times compared to the other experimental groups with significant differences ($p \leq 0.05$) when compared to free FO. These data point to the capacity of this nanocarrier to improve the bioavailability of FO, which is a highly lipophilic compound, regardless of the dietary condition, which is an important characteristic for this type of nutritional supplementation for oral administration.

It is also important to note that the FO samples were kept in the stomach for at least 24 h, while NEW3 was removed from the stomach very quickly (~ 75 % in two hours). These data suggest that fish oil retention in the stomach can be significantly reduced. This finding may be useful to improve possible gastric discomforts associated with oral intake of conventional fish oil capsules, a problem that is often cited by many consumers.

In addition, this study has a strong correlation with the bioavailability of PUFAs [62] and can be used as possible evidence of absorption. The absorption dynamics of NEW3 is not significantly altered when the animals are fasting or fed; however, the dynamics of conventional FO is slightly shifted to the left in the group of animals fed, confirming that this type of nutrient is strongly influenced by the quality of diet.

A critical point in the PUFA literature always demonstrates that DHA and EPA are not effectively absorbed when administered orally. The bioavailability of these compounds can be affected by other nutritional aspects, mainly related to the fat content in the diet [63,64]. In addition, supplementation of FO in its conventional form is generally an unpleasant experience, which can impair an individual's adherence to this practice.

The study by Dey et al. [65] shows the comparison of intestinal absorption of fish oil rich in EPA and DHA in a nanoemulsion (NE) and a conventional emulsion (CE) using the in-situ model of single-pass perfusion (infusion of single pass) in rats. In this work, the NE significantly improved the absorption of lipids compared to the CE in the small intestine of rats, reaching a percentage above 45 % for NE compared to 20 % of the CE in the rate of absorbed lipids.

In the upper intestinal tract, water-soluble lipases are present in excessive amounts. Thus, the greater the available lipid and water interface, the greater the number of lipases that will be able to catalyze the hydrolysis of lipids at the lipid-water interface. This interface is increased by the peristaltic movements of the intestine; thus, for a certain period of time, greater amounts of fatty acids can be absorbed [66].

These observations may explain the fact that lipid droplets in the form of nanodroplets can be rapidly hydrolyzed, approaching the lipid absorption site with greater speed, where they pass through the water layer more easily. They can in turn be absorbed by passive diffusion in the intestinal epithelia [67,68].

In this context, the NEW3 nanoemulsion differential is based on the transport of fish oil tricialglycerols (TAG) in an aqueous medium where the absorption process of fat-soluble nutrients (DHA and EPA) is achieved in the intestinal cells. In addition, the formulation contains BBS oil that protects the nanoemulsion from possible oxidative effects (Fig. 2) and presents concentrations of physiologically biocompatible DHA and EPA that can facilitate absorption in cell membranes (Fig. 3).

The advantages of oral supplements offered by means of a nanoemulsion can facilitate the solubilization of fatty acids, as well as improving the absorption potential, thus providing a better fluidity of the membrane [67]. In this sense, the key point of the present study presents the FO transported in a stable long-term NEW3 system, with acceptable concentrations and easily absorbed in membrane phospholipids, therefore, minimizing the exacerbated production of high potential inflammatory mediators.

3.6.2. LPS-induced sepsis model

After the NEW3 nanoemulsion absorption experiments, we carried out the first inflammatory experiment in order to evaluate the inflammatory modulation provided by the supplementation with the NEW3 nanoemulsion and the conventional FO. For this, the experimental animals received a diet with different treatments for 21 days.

Soon after this period, the animals were submitted to an inflammatory challenge where they received a subcutaneous injection of LPS, used to simulate Sepsis. For this challenge, we measured neutrophil infiltration using the *in vivo* imaging technique at 2 h and 24 h after induction, and the survival profile of experimental mice up to 48 h is shown in Fig. 5.

Fig. 5**A** shows the infiltration of neutrophils after 2 h in animals treated with NEW3, FO and sunflower oil (SO). Sunflower oil supplementation was used because of its composition in omega-6 polyunsaturated fatty acids. In the graph we can see that this time interval was not enough to produce significant differences between the experimental groups. However, when measuring after 24 h (Fig. 5B), we observed a significant increase in neutrophil infiltration in animals treated with SO compared to the other groups. As this oil is a source of omega-6 PUFAs that will produce pro-inflammatory eicosanoids, we hoped that this supplementation could increase inflammation. Fig. 5**C** shows the detected signals of fluorescence *in vivo* in the different experimental groups.

In Fig. 5D, we present the survival profile. As can be seen, 20 % of the experimental mice in the control group survived until the end of the trial, while 80 % of the mice treated with NEW3 and FO survived. SO treated animals had a survival rate of 60 %. The survival profile between these groups was significantly different: interestingly, after the first two hours of the infection induced by sepsis there was a significant death rate among the animals in the control group that did not receive treatment, even not observing differences in neutrophil infiltration, leaving only 1 surviving animal 48 h after the end of the test.

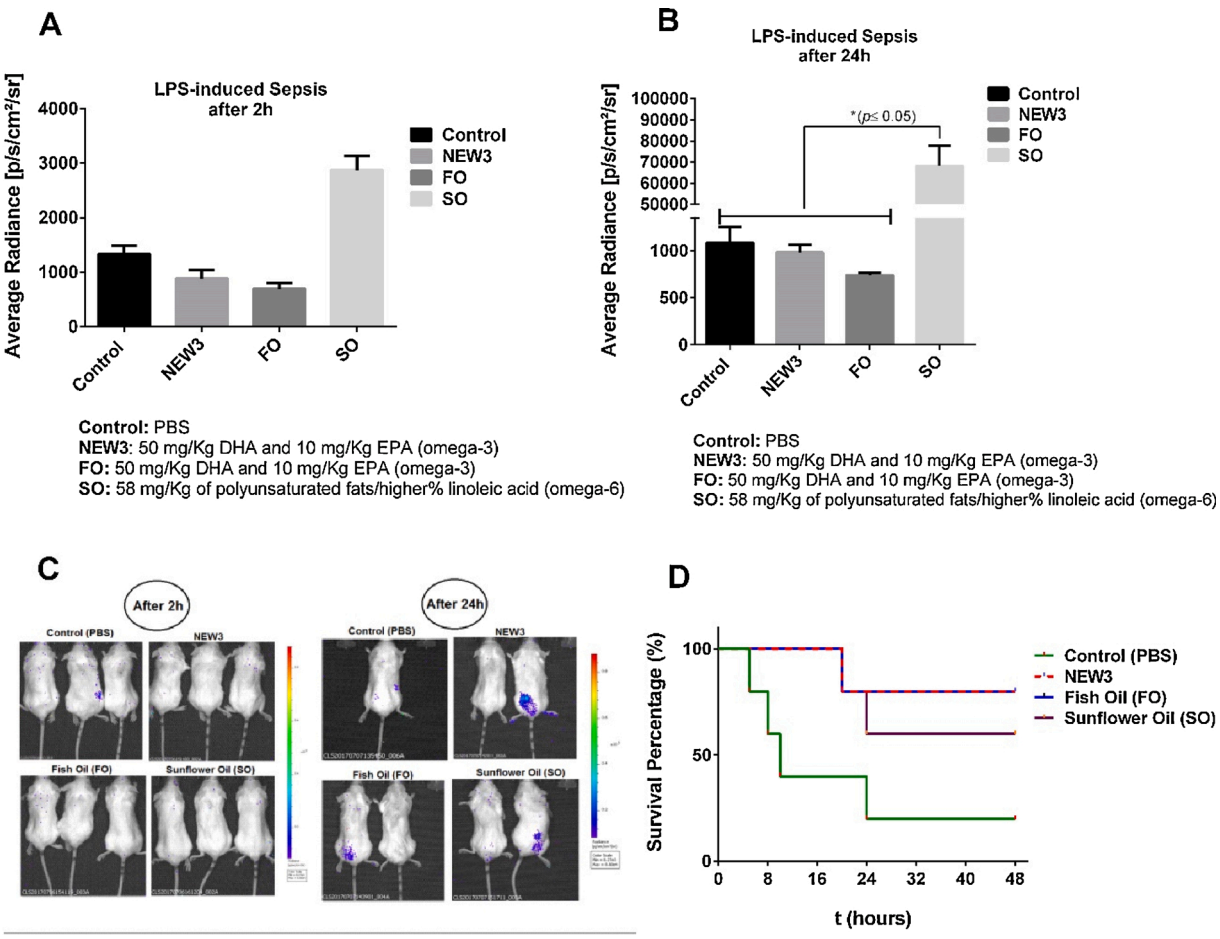

Fig. 5. Inflammatory challenge LPS-induced sepsis. **(A)** average radiance of neutrophil inflation after 2 h; **(B)** average radiance of neutrophil inflation after 24 h; **(C)** qualitative image of fluorescence after 2 h and 24 h; **(D)** Survival profile (%) up to 48 h after sepsis induction. All animals received a dose of 2 mg/Kg of LPS. The control group during the supplementation period was not treated, receiving PBS. Both the NEW3 and FO groups were treated with a daily dose of fish oil in the sample of 100 mg/Kg (containing 50 mg/Kg of DHA and 10 mg/Kg of EPA). The sunflower oil group was treated with a daily dose of sunflower oil of 100 mg/Kg (containing 58 mg/Kg of linoleic acid). **Legends**: **NEW3**: fish oil nanoemulsion; **FO**: fish oil; **SO**: sunflower oil; **t**: time. The data are plotted according to the mean ± SD, the multiple comparison test ANOVA, Tukey test was applied adopting the significance level of $*p \leq 0.05$.

In *in vivo* models, sepsis is achieved when the animal has an infection and demonstrates toxicity or other signs of the disease, and when untreated it can quickly die [68]. The high survival rate for the groups treated with NEW3 and FO may signal a protective effect against the induced bacterial infection. In this work, we selected an inflammatory initiator that triggers the inflammatory process through different biological pathways. We used lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, which is a potent activator of neutrophils and monocytes [69]. In our experimental conditions, after cell activation, a cytokine storm is released and LPS sepsis is installed [70]. We use this model because FO supplementation has been used to prevent sepsis in patients with severe high-risk burns in the ICU [2].

Omega-3 fatty acids DHA and EPA compete with omega-6 fatty acids, that is, with arachidonic acid (AA) to produce anti-inflammatory mediators in the COX-2 pathway. In this enzymatic competition, DHA and EPA will significantly reduce the extent of inflammation [71]. It is important to note that both groups containing fish oil (NEW3 and FO) had interesting survival profiles, signaling that this type of supplementation can be useful to improve the survival rates of patients in the ICU.

In the study by Svahn et al. [72], animals fed an omega-3 fatty acid diet had a longer survival time and a lower bacterial load compared to those fed with saturated fatty acids, after sepsis induced by *S. aureus*. These results highlight that the associations between nutritional factors, lipid profile and survival are of considerable interest in interventions for serious bacterial infections.

Some studies have demonstrated lipid emulsions containing fish oil for severe septic patients as part of parenteral nutrition [73]. However, there are not yet enough studies of fish oil-based nanoemulsions as part of the oral diet for prevention or therapeutic measure for sepsis. We tested the NEW3 nanoemulsion where we observed promising prophylactic effects for sepsis in an *in vivo* experimental model. Therefore, to further explore the inflammatory modulation of NEW3 supplementation, we performed the second inflammatory challenge, as demonstrated in the next item.

3.6.3. *Formalin-induced inflammation model paw edema*

The second challenge of *in vivo* inflammation investigated was the formalin-induced paw edema model, as shown in Fig. 6. In Fig. 6A, we observed the kinetics of the volume of the paw edema in the different experimental groups. Only the NEW3 group showed a significant reduction in edema volume ($p \leq 0.05$) during the experiments. In all other experimental treatments, a significant increase in edema volume was observed. Fig. 6**B** represents the area under the curve (AUC) of the experimental data.

Edema induction can be done with agents that lead to a local inflammatory response [74,75]. for this experiment we used Formalin (FM) which is a highly reactive compound that can produce cross-links between cellular proteins [75,76]. In addition, this molecule can reduce cell glutathione activity, leading to the generation of reactive oxygen species (ROS) [77]. ROS in turn damage cellular structures, thus

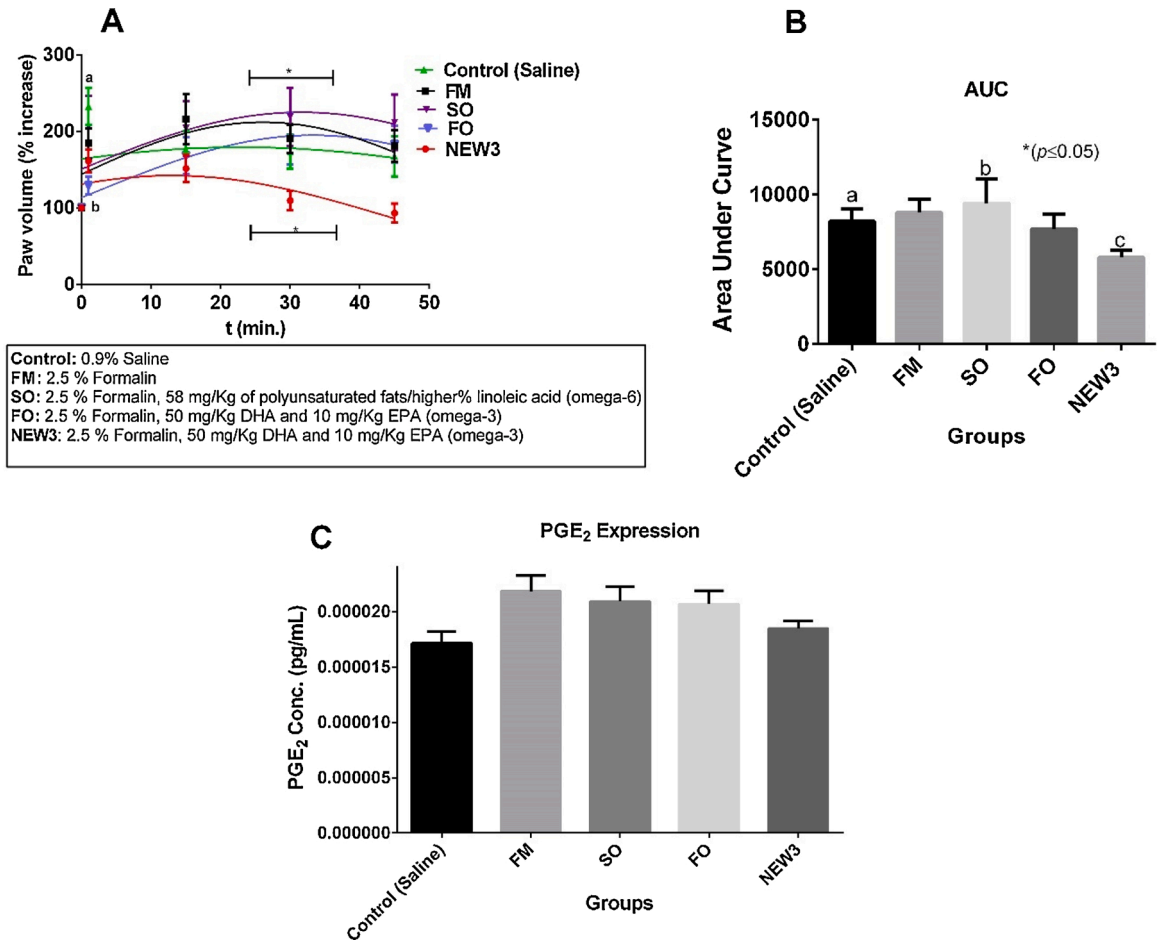

Fig. 6. Paw edema inflammation model induced with formalin. **(A)** percentage of the increase in the paw volume of the experimental groups; **(B)** area under the experimental data curve; **(C)** concentration of prostaglandin PGE_2 (pg/mL) in the tissue of the animals' right paws. All animals were induced with an injection of 2.5 % formalin, except for the saline control group, which was induced with 0.9 % NaCl. The saline control group and the formalin group during the supplementation period were not treated, receiving only PBS. Both the NEW3 and FO groups were treated with a daily dose of fish oil in the sample of 100 mg/Kg (containing 50 mg/Kg of DHA and 10 mg/Kg of EPA). The sunflower oil group was treated with a daily dose of sunflower oil of 100 mg/Kg (containing 58 mg/Kg of linoleic acid). **Legends: NEW3**: fish oil nanoemulsion; **FO**: fish oil; **SO**: sunflower oil; **t**: time; **PGE_2**: prostaglandin series 2. Data are plotted according to the mean ± SD, the multiple comparison test ANOVA, Tukey test was applied adopting the significance level of $p \leq 0.05$. Different letters represent statistical differences *($p \leq 0.05$).

triggering a local inflammatory process.

As shown in Fig. 6A, the volumes of the animals' paw edema were less pronounced for the group treated with the NEW3 nanoemulsion, compared to the other groups. More intense edema was also observed in animals supplemented with sunflower oil (SO) (Fig. 6B), confirming the data found in the first inflammatory challenge. The animals supplemented with fish oil (FO) showed similar results to the control group, FM and SO, with significant differences in the percentage of increased paw volume when compared to the NEW3 treatment (Fig. 6A).

This event also allows the intensity of the nociceptive response to be analyzed in two phases: acute and late, both with different properties. The initial phase comprises from zero to five minutes; it is called non-inflammatory (neurogenic) pain and reflects central pain. The second starts 15−30 min after the injection; it is called inflammatory pain and is characterized by local inflammation from the release of inflammatory mediators such as prostaglandins (PGE_2) and hyperalgesia [74,75].

It is noted that in the time interval characterized by inflammatory pain a milder decreasing curve was observed for the group treated with NEW3 compared to the other groups (Fig. 6A), indicating here a biological effect of the NEW3 nanoemulsion dosage containing 100 mg/Kg of fish oil with 50 mg/Kg of DHA and 10 mg/Kg of EPA.

There was no significant effect of the FO group in the same amounts administered in relation to the control group, FM and SO, 15 min after the injection of Formalin. These results suggest that the dosage of fish oil was more effective in the NEW3 nanoemulsion to reduce formalin-induced paw edema, pointing here to anti-inflammatory activity.

The reduction of edema in mice is one of the most suitable procedures for screening anti-inflammatory agents [74,75]. This assay is considered an appropriate experimental model for the evaluation of the acute anti-inflammatory effect of various agents [78]. Specifically, for fish oil, some studies demonstrate the anti-inflammatory and analgesic effects evaluated by the paw edema method [79]. However, they are studies using samples of conventional oils and not nanoemulsified.

In this experiment, we also measured the concentration of PGE_2 in the tissues of the paw collected from the experimental groups, PGE_2 is a prostaglandin characterized as a biomarker of inflammation. During edema a cascade of biochemical events occurs and propagates an inflammatory response involving the migration of various blood leukocytes to the site of infection or edema. Leukocytes drive the release of mediators at the site of inflammation, which may include lipid mediators such as prostaglandins (PG) and leukotrienes (LT) [10,11,17].

As shown in Fig. 6C, this biomarker presented a slight reduction in animals treated with NEW3 compared to animals in the FM group; however, this reduction was not statistically significant. No significant difference was observed when comparing all other experimental groups. These results indicate that the inflammatory challenge, by the injection of an initiator such as formalin, induced the expression of PGE_2 at detectable levels that were not differentiated in the statistical tests.

However, the small decrease in PGE_2 expression, together with the significant reduction in paw edema in animals supplemented with NEW3 nanoemulsion, may indicate possible biological protection in the response of the acute phase of an inflammatory process.

3.6.4. Hematological and histological analysis

The results of the hematological analysis for the erythrogram, total platelet count and leukogram of the experimental groups of the paw edema model are shown in Table 2. The data analysis shows that there were no significant changes ($p \leq 0.05$) in the blood cells in the analyzed parameters among the groups tested.

Hematological evaluation is an important analysis for determining the physiological profile of animals and is commonly used for investigations in response to genetically induced infectious diseases or by dietary and pharmacological therapies. In addition, it helps in the creation of reference values that reflect a given health condition of the animal [80]. However, establishing reference ranges for rodents requires greater accuracy, since there are several important guidelines that must be considered that range from the animal's genetic variation to the environmental conditions in which these animals live [81]. The values expressed for hematological parameters for NEW3 showed similarities with data from the literature [81,82].

When measuring the masses of the livers for the different experimental groups of the paw edema model, there was only a slight ($p \leq 0.05$) divergence in the mean of the NEW3 group compared to the saline control group. However, this mean did not differ significantly ($p \leq 0.05$) among the other groups, and no major macroscopic visual changes were observed in the experimental groups (**Supplementary Fig. S5**).

In the histological analysis of the livers of the animals submitted to the different treatments, we observed that for the FM group (formalin) there were microscopic changes in the structure of the liver tissue, with inflammatory foci and brief indications of hemorrhages and hepatic necrosis, as expected. For the saline control group there were no changes in the tissue structure: the FO (fish oil) group had few inflammatory infiltrates, mainly around vessels; the SO (sunflower oil) group had small inflammatory foci with changes in the structure of the liver tissue. For the NEW3 group, small inflammatory infiltrates were observed, but there was no pathology in the tissue.

All of these outcomes are important observations, demonstrating that the treatment with NEW3 was well tolerated and providing responses that did not affect the quality of the animals' health during the study period investigated.

3.6.5. Clinical aspects of treatments

In order to know if the different treatments would be toxic to the animals during the period of supplementation that preceded the inflammatory challenges, the evaluation of some clinical parameters was carried out, and we organized these results in Fig. 7. Fig. 7**A** and **7B** shows the average of feed consumption per group for the two experimental lots destined for the sepsis and paw edema models, respectively. It is noted that there was a gradual increase in feed consumption per group for animals representative of the 1 st experimental batch, while in the 2nd batch a constant was observed between the groups with differences ($p \leq 0.05$) in consumption, and a significant decrease ($p \leq 0.05$) for the SO group.

As explained at other times, the animals received an oral supplementation of NEW3, FO and SO for 21 days, before the inflammatory challenges, so this is an important period in which to evaluate the possible toxicological activities as well as the clinical aspects of these treatments. In Fig. 7**C** and **D**, the animals' body mass averages are presented; no differences were observed in the change in the animals' weights in relation to the control group (Fig. 7C), and the initial weight range of the animals in the first batch was around 22.0–25.0 g.

For the animals obtained for the second batch, the initial weight range for each group was: saline control (16–20 g), FM (20–21 g), SO (22–23 g), FO (23–26 g) and NEW3 (26–31 g). This grouping was necessary since the batch of animals supplied did not present uniformity, but it can be observed that during the experiment the means of the groups were still within these ranges Fig. 7**D**.

The data referring to individual weight gain (Fig. 7**E** and **F**) and the feed efficiency determined by means of the food efficiency coefficient (CEA) (Fig. 7**G** and **H**) showed that, for each treatment, regardless of batches, significant differences were not observed between groups. The animal's metabolism can be affected by different causes and also by environmental conditions. It can be noted that the treatments with the NEW3 nanoemulsion and the other experimental groups for 21 days in two different periods did not affect the main murinometric measures, demonstrating the reproducibility of the experimental method of oral supplementation of these formulations and also indicating biocompatibility of the tested dosage of the formulation NEW3 in an animal model.

4. Conclusion

In conclusion, we observed that the NEW3 nanoemulsion developed in this study showed long-term colloidal and chemical stability. NEW3 was useful in reducing the gastric emptying time of lipophilic compounds such as fish oil and, therefore, can be an important formulation to reduce gastric discomfort related to oral intake of fish oil capsules. NEW3 proved to be an interesting oral nanocarrier for administering fish oil and showed results with protective effects on animals when tested in two different inflammatory experiments. The inflammation models and the experimental method of supplementation were reproducible and offered similar results.

Table 2
Erythrogram and Leukogram of Balb/c mice submitted to different treatments after induction of the paw edema inflammation model.

Erythrogram						
Parameter	Unity	Control	FM	SO	FO	NEW3
RBC	$x10^6/\mu L$	14.16 ± 1.48	12.68 ± 1.21	14.69 ± 0.88	7.04 ± 5.27	5.98 ± 4.93
HGB	g/dL	20.2 ± 2.11	16.78 ± 2.49	20.77 ± 1.67	9.92 ± 7.32	13.85 ± 14.39
HCT	%	53.54 ± 5.75	47.61 ± 4.20	54.68 ± 4.82	25.55 ± 19.01	19.96 ± 18.38
PTL	$x10^3/\mu L$	786.6 ± 159.18	1350.66 ± 736.40	1040.71 ± 202.25	977.66 ± 369.85	1688.66 ± 2619.93
Leukogram						
Parameter	Unity	Control	FM	SO	FO	NEW3
WBC	$x10^3/\mu L$	3.98 ± 2.19	7.42 ± 6.38	4.07 ± 1.28	2.82 ± 1.77	5.13 ± 4.05
W-SCC	$x10^3/\mu L$	2.34 ± 1.28	3.77 ± 2.97	2.1 ± 1.21	1.92 ± 0.81	3.32 ± 2.17
W-MCC	$x10^3/\mu L$	1.48 ± 0.79	3.23 ± 3.36	1.83 ± 0.41	1450 ± 369.68	2.48 ± 2.03
W-LCC	$x10^3/\mu L$	0.16 ± 0.11	0.42 ± 0.55	0.13 ± 0.18	125 ± 95.74	0.36 ± 0.27

Control: Saline; FM: Formalin; SO: sunflower oil; FO: Fish oil; NEW3: Fish oil nanoemulsion; RBC: total red blood cell count; HGB: hemoglobin concentration; HCT: hematocrit percentage; PTL: platelets count; WBC: total white blood cell count; W-SCC: absolute small white blood cell count - lymphocytes; W-MCC: intermediate cell count – monocytes; W-LCC: large cell count – neutrophils.

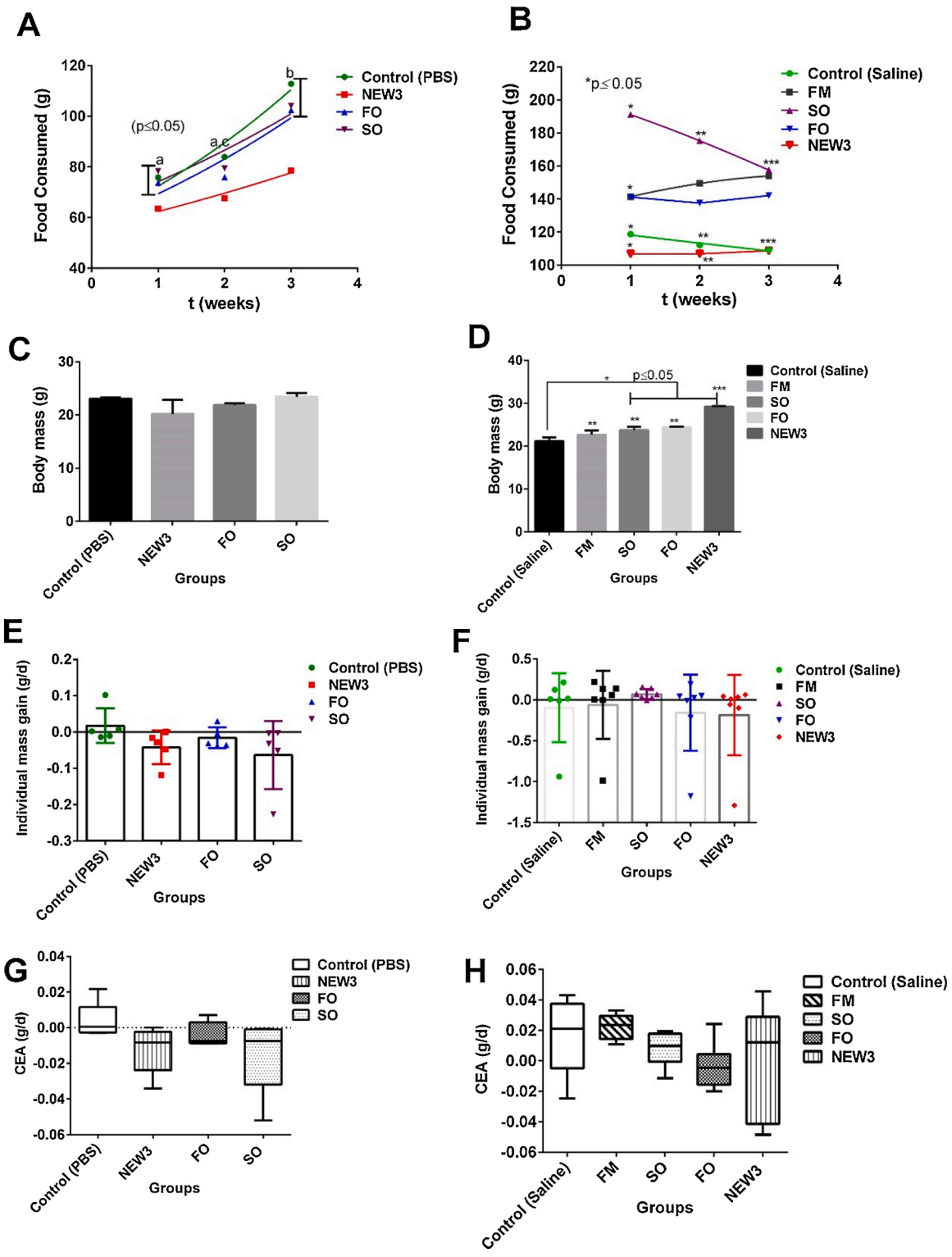

Fig. 7. Murinometric measurements of adult female balb / c animals submitted to oral treatments with NEW3, FO and SO supplementation. **(A)** and **(B)** feed consumption by group (g); **(C)** and **(D)** Body Mass (g); **(E)** and **(F)** Individual weight gain (g/d); **(G)** and **(H)** food efficiency coefficient - CEA (g/d). The animals were supplemented with the following dosages in two different periods for 21 days: The saline control group and the formalin group were not treated, receiving only PBS. The NEW3 and FO group were treated with a daily dose of fish oil in the sample of 100 mg/Kg (containing 50 mg/Kg of DHA and 10 mg/Kg of EPA). The sunflower oil group was treated with a daily dose of sunflower oil of 100 mg/Kg (containing 58 mg/Kg of linoleic acid). (A, C, E and G) refers to the first experimental batch for the Sepsis model, (B, D, F and H) refers to the second experimental batch for the paw edema model. **Legends: NEW3**: fish oil nanoemulsion; **FO**: fish oil; **SO**: sunflower oil; **t:** time. The data are plotted according to the mean ± SD, the multiple comparison test ANOVA, Tukey test was applied adopting the significance level of $*p \leq 0.05$.

Financial & competing interests disclosure

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110980.

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